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Protease

The present invention relates to a protease, nucleic acids coding for the protease, and the related inhibitors, antibodies, medicaments and diagnostic agents.

The present invention provides a protease having two aspartate residues in a catalytically active structure, wherein a first aspartate residue resides in an X_1GX_2GD motif and a second aspartate residue resides in an $X_3X_4DX_5$ motif, wherein X_1, X_2, X_3 and X_5 are independently selected from Ala, Val, Leu, Met and Ile, and X_4 is an aromatic amino acid, and the motifs X_1GX_2GD and $X_3X_4DX_5$ reside in a transmembrane region. For the motifs, the one-letter code of amino acids has been used, i.e., D = Asp, G = Gly, etc.

There is a high probability that such proteases are involved in the cleavage of the amyloid precursor protein (APP). In one embodiment of the invention, the protease according to the invention is the as yet unidentified γ-secretase, which is involved in the processing of APP into the amyloid peptides referred to as Aβ.

A survey of the role of γ-secretases in the genesis of Alzheimer's disease is given by S.L. Ross et al. in J. of Biol. Chem. 273 (1998), 15309-15312.

Preferred proteases of the present invention additionally have a sequence $PALX_6YX_7V$, wherein X_6 and X_7 independently have the same meaning as X_1. However, it is preferred that X_6 and X_7 are leucine or isoleucine.

In particular, the proteases are proteases from mammals, especially humans.

The proteases according to the invention preferably have catalytically active aspartate residues in a region which lies within a transmembrane domain. When the sequence of a protein is known, transmembrane domains can be predicted due to different models. They are characterized by the fact that hydrophobic amino acids are predominant in a region which is flanked by regions in which hydrophilic amino acids are predominant.

An aspartate in a transmembrane region can be detected, for example, by the application of the program "GREASE" which is a part of the FASTA 2.0 program package. For a window width of 17, a hydrophobicity value of at least 80 must be calculated for the aspartate using this program. The FASTA program package has been described in W.R. Pearson and D.J. Lipman, Proc. Natl. Acad. Sci. U.S.A. 85 (1988), 2444-2448. The program GREASE makes use of the Kyte/Doolittle algorithm, which has been described in J. Kyte and R.F. Doolittle, J. Mol. Biol. 157 (1982), 105-132.

Particularly preferred proteases of the present invention are referred to as psl 1-5 (human psl 1-5: SEQ ID Nos. 1 to 4 + 19; murine psl 2-4: SEQ ID Nos. 5-7; *Sacc. cerevisiae* psl 3: SEQ ID No. 8; human psl2L SEQ ID No. 18).

The invention further relates to variants of the proteases according to the invention. Variants are proteins which are derived from the proteases according to the invention by one or more mutations, insertions and deletions, especially by conservative substitutions, in particular, forms truncated or extended on the N or C terminus.

The invention also relates to nucleic acids coding for the proteases according to the invention. Preferred nucleic acids according to the invention are those having the SEQ ID Nos. 9 to 17 + 20 (human psl 1-5: SEQ ID Nos. 9 to 12 + 20; murine psl 2-4: SEQ ID Nos. 13-15, *Sacc. cerevisiae* psl 3: SEQ ID No. 16, human psl2L SEQ ID No. 17). Complementary nucleic acids are also part of the invention.

The proteases according to the invention are involved in the cleavage of APP into Aβ and are thus indirectly involved in the genesis of, for example, Alzheimer's

disease. Therefore, the invention also relates to inhibitors which inhibit the expression or activity of the proteases. Such inhibitors can be identified in simple methods. Appropriate inhibitors can be detected, for example, by measuring the expression or activity of the proteases in the presence of potential inhibitors. Antibodies directed against the aspartate proteases are particularly suitable for measuring the expression and are thus also part of the invention.

The aspartate proteases according to the invention are also involved in the cleavage of other transmembrane proteins, especially of the receptor protein Notch and related proteins which play a role in the development of the nervous system.

The proteolytic cleavage in the interior of membranes is also involved in other important processes, e.g.:

- Proteolytic degradation of N-terminal signal peptides after their cleavage by signal peptidase.

- Proteolytic degradation of C-terminal propeptides such as those formed by the transamidase-catalyzed cleavage in the posttranslational GPI anchoring of proteins.

- Generation of peptides for the presentation by histocompatibility complex molecules of types I and II. For soluble proteins, such peptides are predominantly produced by the proteasome. However, peptides are also formed from transmembrane regions of proteins which can only be explained by a cleavage in the interior of the membrane.

- Proteolytic cleavage of the ER stress sensor protein Ire1. The endoplasmic reticulum possesses a mechanism which detects the accumulation of unfolded or incorrectly folded proteins and sends a signal into the nucleus. This mechanism is referred to as "unfolded protein response" or UPR and results in the increased formation of proteins which facilitate the folding. In mammals, there are two sensor proteins (Ire1alpha and Ire1beta) which respond to folding defects and are then proteolytically cleaved within the membrane.

By using the protease according to the invention or its inhibitors, the mentioned processes can be influenced therapeutically.

Useful cell lines include those, in particular, which do not express any of the proteases or contain any of the nucleic acids according to the invention, and preferably those which do not contain any homologous proteases or nucleic acids either. Preferably, they can be used to test the activity of the proteases according to the method described in Example 1 or to establish inhibitors according to Example 2. Particularly suitable is *Saccharomyces cerevisiae*. When the corresponding protease or the nucleic acid coding for it (SEQ ID Nos. 8 and 16) is known, yeast strains which no longer contain this protein or the nucleic acid can be produced by known methods. Therefore, they are preferably suitable as an expression system for the characterization of the aspartate proteases according to the invention or for the identification of suitable inhibitors. Therefore, the invention also relates to the corresponding cell lines, preferably yeast cell lines, and to the use of the protein of SEQ ID No. 8 as an aspartate protease, and of the nucleic acid of SEQ ID No. 16 for the expression of a protease.

The proteases, nucleic acids, inhibitors and antibodies according to the invention can be contained in medicaments and diagnostic agents. They are suitable, in particular, for the treatment and diagnosis of diseases which are causally related with the cleavage of the amyloid precursor protein, especially Alzheimer's disease.

Example 1:

γ-secretase assay

The putative γ-secretases are stably or transiently transfected into cos-7 cells which additionally stably express SpA4CT (signal peptide fused to βA4, followed by APP C-terminus). In this system, γ-secretase activity can be detected by the generation of a 4.6 kDa peptide or by the disappearance of the 11 kDa band of the complete SpA4CT. When the pathologically relevant γ-secretase is present, both fragments should be detectable in the interior of the cell. In the supernatant of the cells, βA4 can always be found; it is generated by an endogenous γ-secretase

activity residing in the plasma membrane, but which does not play a role in the pathogenesis of Alzheimer's disease.

The transfected cells are washed three times with cold DMEM and subsequently harvested on ice with a squeegee. The cells (about 5 x 10^6 cells) are collected by centrifugation and lysed in 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% NP-40, 1% Triton X-100, 2 mM EDTA). The nuclei are centrifuged off at 11,000 x g. The supernatant is subjected to immunoprecipitation. Thus, 1 ml of the cell lysate is mixed with 2 µg/ml of WO2 immunoglobulin (anti-βA4 antibody) and shaken upside down at 4 °C for 0.5 h. Subsequently, 20 µl of protein G sepharose suspension (1:1) is added, followed by shaking upside down at 4 °C for 5 h. The protein G sepharose is successively washed twice with buffers A, B and C (A: 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.2% NP-40, 2 mM EDTA; B: 500 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.2% NP-40, 2 mM EDTA; C: 10 mM Tris-HCl, pH 7.5), mixed with 20 µl of 3x sample buffer, heated to 95 °C, and the supernatant is coated onto a 12% Tris-Tricine gel. After gel-electrophoretic size fractionation, the proteins are transferred to a PVDF membrane and subsequently detected with an anti-βA4 antibody.

Example 2:

Identification of a γ-secretase inhibitor

For the identification of an inhibitor of the pathologically relevant γ-secretase, the enzyme is co-expressed with SpA4CT in cos-7 cells according to the above protocol. The cells are suitably contacted with the substance to be examined (in the presence or absence of membrane-permeabilizing agents). Subsequently, the intracellularly formed βA4 is detected as described above. A reduction of the quantity of βA4 formed permits to conclude the activity of the substance as a γ-secretase inhibitor.